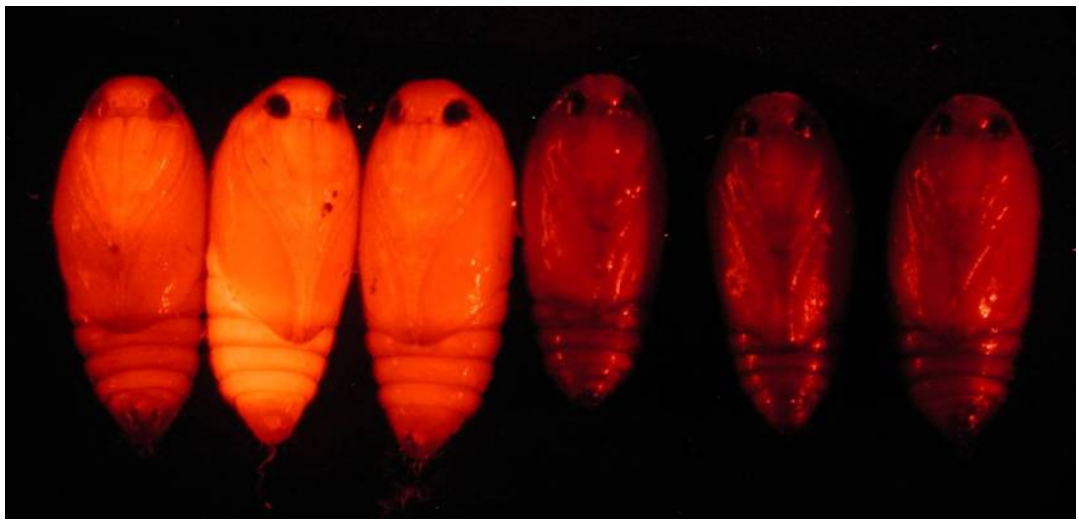
Molecular Diagnostics & Biotechnology National Science Program

Center for Plant Health Science & Technology



Annual Report 2004

USDA

APHIS

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Molecular Diagnostics and Biotechnology

Introduction

The Molecular Diagnostics and Biotechnology (MDBT) staff's mission is to provides scientific support to APHIS PPQ regarding a broad range of issues related to molecular diagnostics and genetically modified organisms.

The MDBT program has three main focus areas. First, the team identifies, develops, or adapts appropriate state-of-the-art technologies for molecular-based detection and identification of agents of interest to APHIS PPQ. Molecular detection methods may be based on nucleic acids or proteins, but may also utilize immunological or biochemical methods. Additionally, laboratories are responsible for validating molecular assays that are destined for use by the regulatory community. Finally, the team provides scientific support for the development of genetically modified insects for the control of crop pests.

Underpinning our activities is a strong science base. Our vision is to identify and employ the best and most effective technologies available to ensure that APHIS PPQ delivers effective programs. Assays that are developed must be highly reproducible and robust. Similarly, regulation of deployment of GM organisms must be based on sound science.

Technologies are evaluated for their potential to improve APHIS PPQ program management. Program activities include:

- Obtain, evaluate or develop state-of-the-art technologies for molecular diagnostics of exotic pests.
- Validation of molecular diagnostics.
- Provide training to other Federal or State laboratories on the use of specific molecular diagnostics.
- Provide Quality Assurance of accepted, validated molecular diagnostics.

Profile of an MDBT program:

> Pink bollworm (PBW) is one of the most destructive insects of cotton, costing U.S. farmers over $30 million per year. One of the key control strategies is to mass release sterilized male insects to interrupt reproduction. These insects are reared in specialized facilities and sterilized by irradiation. We are investigating the use of genetically modified insects that contain a conditional lethal mutant gene. When these insects are released, it is hoped that they will effectively suppress PBW populations, resulting in reduced costs and increased efficiency of using this control strategy.

The adaptation and adoption of new technologies so that APHIS PPQ can meet its goals is a primary function of the Molecular Diagnostics and Biotechnology Program. The National Plant Germplasm and Biotechnology Laboratory (NPGBL), Beltsville, MD, performs applied research development of molecular diagnostics for regulated plant pests. Researchers at the NPGBL use state-of-the-art technology to validate and/or improve existing diagnosti methods, and they develop methods when none exist. This facility also screens foreign germplasm introductions for exotic pests.

Other CPHST laboratories perform/develop molecular diagnostics for specific insects (e.g. Medfly and Mexfly) to support APHIS PPQ regulatory programs. These activities provide key support for regional and state plant disease diagnostic laboratories, plant introductions, border inspections, and international trade. The Molecular Diagnostics and Biotechnology group is also working on the development and utilization of genetically modified insect pests for implementation of improved control strategies.

PBW Eradication Support, EGFP Release and Pheromone Tech Transfer

Robert Staten

Decision Support & Pest Management Systems Laboratory, Phoenix, AZ

Budget cuts restricted CPHST resources for EGFP release within the planned eradication zones. All strategies for release will have to be reconsidered.

Treatment	Bolls/field	Larvae/field
ROPE	564.67	6.78*
COIL	550.22	11.78

Figure 1. HIGH RATE PHEROMONE SYSTEMS COMPARISION 2004 (BOLLBOX DATA)

Major effort in pheromone system development provided data to place a revised high rate system in proper perspective. An 18 field season-long test clearly demonstrated differences between our standard, PBL Rope, and the candidate Spiral formulation. The Spiral does not compete (Fig. 1).

Development/screening of "sprayable" systems continued. We tested materials from Thies Technologies and Pherotech (Israel). Standards have not been beaten in full-field trials. Neither gave the longevity required. A four-replicate test of 40 acres each of a Suterra sprayable vs. Sentry standard was also disappointing. However, two materials, Shin Etsu and ISCA Technology, show promise and will be pursued extensively. Should one of these two materials work as well as they appear it will shift eradication cost equations significantly (Figs. 2 and 3).

Sterile release was managed, courtesy of California Cotton Growers, on an experimental basis on 5,000+ acres in New Mexico and 10,000+ acres in Texas. Valuable information was acquired relative to what will be required for continuation on 80,000+ acres in 2005.

Within the Texas release zone one area isolated by 30+ miles from any cotton and 50-60 miles from all programmatic cotton

Figure 2."Sprayable" wax PBW pheromone, SPLAT, in the field.

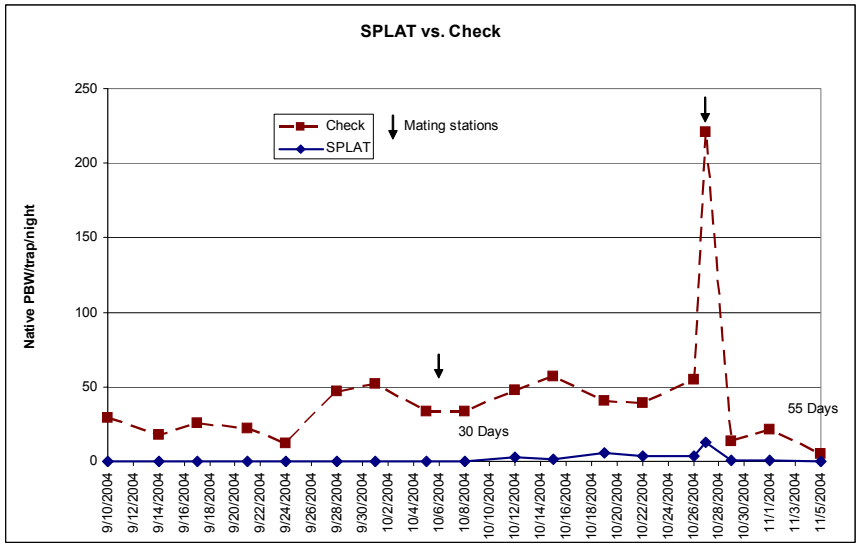


Figure 3. SPLAT strongly outperformed the check during very high fall populations of PBW, 2004.

was grown with no conventional cotton as refugia. Sterile insects were used as refugia. No larva was found in 11 miles of rosetted bloom survey and in 13,000 late season bolls. Migration was very apparent at the end of season.

In support of future releases of transgenic insects, a test comparing the hostability of okra vs. cotton was conducted using 24 paired plots of okra. Infestation of cotton bolls by larva was 11.6 times higher than okra when PBW is directly introduced in mixed plantings. This information will be useful in future risk assessments for transgenic insects.

COLLABORATORS: Arizona Cotton Research and Protection Council, California Department of Food and Agriculture, Texas Boll Weevil Eradication, and New Mexico Boll Weevil Eradication

PROJECT DELIVERABLES

- Provided critical data to support pheromone purchase in the PBW Eradication Program in 2005
- Identified 2 highly promising "sprayable" pheromone systems for PBW Eradication Program
- Sterile release provided population suppression in 2004 and vital data for conduct of the 2005 program.
- We provided data which may be used to support 100% Bt cotton areas within an eradication program

Development and Implementation of new procedures for use in the Pink Bollworm Rearing Facility (PBWRF) to reduce production costs and improve insect quality

Ernie Miller

Decision Support & Pest Management Systems Laboratory, Phoenix, AZ

PROJECT HIGHLIGHTS

The CPHST development work on new technologies for mass rearing PBW has resulted in the transfer of two new technologies to the Pink Bollworm Mass Rearing Facility, located in Phoenix, AZ. These changes, once implemented into the program, will reduce rearing costs and improve insect quality and working conditions for insect production workers.

PROBLEM STATEMENT: In its 35 years of existence, the Pink Bollworm Rearing Facility (PBWRF) has never been called on to produce more than 8 million moths per day. However, when Sterile Insect Technique (SIT) is implemented into the Texas Pink Bollworm Eradication Program, daily moth production by the facility for the existing California program and the eradication program is anticipated to be around 30 million. The objectives of the following studies are to develop procedures that could be implemented as standard rearing protocols in the Pink Bollworm Rearing Facility to reduce operational costs and improve insect quality.

The SIT component of eradication and preventive programs is seriously challenged by costs. Therefore, any cost savings that can be realized are critical for the continued utilization of this component in any pest management program.

APPROACH: We targeted two tasks regularly performed by the PBWRF. First was the development of a multipurpose moth shipping/release container to replace the current shipping box and

Corrugated plastic sheet (blue substrate) that will replace cardboard on L. Cardboard on R. Each with honeycomb lying on top. The honeycomb is used as pupation substrate for mass rearing PBW.

separate release machine moth holding box mounted on top of the release machine. This equipment is fitted into an aircraft for the aerial release of PBW. Second was the replacement of the cardboard backing used with honeycomb for the collection of larvae (pupation site) from larval rearing containers within the facility.

MULTIPURPOSE MOTH SHIPPING/RELEASE BOX: In FY04, collaborative field testing of the shipping/release box was conducted with CDFA and APHIS personnel managing the San Joaquin PBW-SIT program. The new shipping/release box with some additional modifications to the outside cover was integrated into the regular program shipping cycle 8 times over the course of the 2004 season. According to standard protocol, the moths were aerially released the following day with the bulk of the moths in the box being released over regular program fields. In addition to the program release fields, two 160 acre cotton fields isolated by at least 2 miles from other sterile release cotton fields in Kern county of the San Joaquin valley were selected, with one field receiving moths shipped in the new shipping release box and the other receiving moths shipped and released using the current Program protocol. This was replicated 8 times over the course of the season. Traps were placed in each field at a rate of 1 trap/10 acres and checked 1-2 times per week by CDFA personnel until mean moth captures fell below 5 moths per trap.

Prototype of dual function box that serves as a shipping container and holding box on the aerial drop machine used in releasing sterile PBW over cotton fields in the San Joaquin Valley SIT Program.

REPLACEMENT OF THE CARDBOARD BACKING USED IN PBW LARVAL COLLECTION: A replacement substrate was selected from preliminary testing of three candidate materials. Several parameters of the material were essential, including a light weight to allow insect production workers to handle 15 -20 pieces at a time. The substrated needed to be bleach resistant to allow sterilization between uses and have a rigid, flat structure so larvae could not crawl under the material once it was in place on the rearing cart. Additionally, the material could not be toxic to the larvae and the surface of the material must be textured enough to allow the permanent adhesion of silk spun by the larva to form one end of its cocoon onto its surface. If the cocoon does not anchor to the material beneath the honeycomb it is nearly impossible to extract the resulting pupae from the honeycomb cell once the two materials are separated during the harvest of the pupae.

ACCOMPLISHMENTS: In 2003, the shipping/release box was bench tested with positive results. Optimal temperatures were maintained for ca. 12 hours and moth mortality was similar to a control group maintained at 40F for the same length of time. A field study comparing moth shipment and release from our new system with the current technology used by the program also provided encouraging results. In 2004, standard program QC testing comparisons of PBW moths shipped in the new shipping/release box and those shipped in the current program shipper indicated no statistical difference in moth quality. However, recapture rates of sterile PBW moths were significantly higher (1.98 times higher) in fields where moths had been released from the new shipping/release box system compared to fields where the current program shipping/release system was used to release moths. Based on the results of this test work, the two PBW-SIT Programs (the San Joaquin project and the Texas/NM/Mexico PBW eradication program) will incorporate the use of this methodology to ship and release their moths. This change will result in improved insect quality and the cost savings within the programs.

Test work in 2003 indicated that the corrugated plastic sheets shown in figure 1 met all the necessary performance requirements needed to integrate this technology into the PBWRF. In 2004, 10% of the PBWRF production used corrugated plastic instead of cardboard. Results over the first 2-1/2 months were consistent with our test work in 2003. However, in late July a problem surfaced. The larval-spun cocoon was no longer anchoring to the plastic material beneath the honeycomb, making it difficult to extract the resulting pupae from the honeycomb cell once the two materials were separated during the harvest of the pupae. Work will continue to solve this problem in 2005. The estimated cost savings in materials for this technology transfer is $12,120 per production year at a 5 million per day production level, and $72,000 per year if the production level of the facility is at 30 million per day, as is anticipated in the near future when the SIT is expanded into NM, TX, AZ and Mexico.

PROJECT DELIVERABLES

- Combining PBW moth shipping and release tasks into a single container reduces the handling of the insects, which improves insect quality and reduces overall program costs.
- Elimination of the transfer of moths from shipping container to release machine negates the need for a moth scale collection system and a walk-in cold room at release site staging locations.
- Replacing the cardboard in PBW larvae collection with reusable corrugated plastic sheets saves $12,000 to $72,000 per production year. Amount of savings is dependent on daily production level.

Evaluating reduced doses of irradiation to improve inset quality in mass reared pink boll worm moths and exploring a transgenic strain as a genetic marker

Ernie Miller

Decision Support & Pest Management Systems Laboratory, Phoenix, AZ

PROJECT HIGHLIGHTS

Pink Bollworm (PBW) male moth quality is improved by lowering the irradiation dose. Under the conditions of our studies a 10 kr dose of irradiation did not produce measurable F1 larval or adult progeny that would be a factor in a Sterile Insect Technique (SIT) program. These data support the use of a 10 kr dose in the PBW-SIT program in lieu of the fully sterilizing dose of 20 kr.

Under the conditions of our tests, the doses of irradiation we tested on female PBW of a transgenic strain did not appear to affect their ability to solicit males from a native field population when compared to irradiated females of a non-transgenic strain of PBW. However, all irradiation doses to females reduced their ability to solicit males when compared to non-irradiated females, regardless of strain type.

PROBLEM STATEMENT: Historically, the approach to managing pink bollworm (PBW) populations in the southern desert valleys of Arizona, Texas, California, and New Mexico was the use of broad spectrum insecticides. More recently a shift in managing PBW pest populations using more target-specific tools has occurred as a result of the success of area-wide use of Bt cotton, pheromone disruption programs, and Sterile Insect Technique (SIT) individually or in various combinations. All are more ecologically sound than the use of insecticides. Our objective is to examine methods that have the potential to enhance the effectiveness of the SIT method in current or future area-wide PBW control and/or eradication programs. Numerous studies have indicated that sterilizing irradiation doses have a major impact on the fitness of released insects in SIT programs. Flint et al, 1973 and Carpenter et al, 2001 have indicated that insects receiving partially-sterilizing doses of irradiation may result in greater population control than those receiving fully-sterilizing doses.

Any improvement in moth quality, particularly improved field-fitness of the release insects, would reduce the overall costs of PBW SIT programs. Improved field fitness would reduce the required high ratio of sterile to wild insects (60:1) that is needed to acquire a high level of sterility in the field. Doing so increases

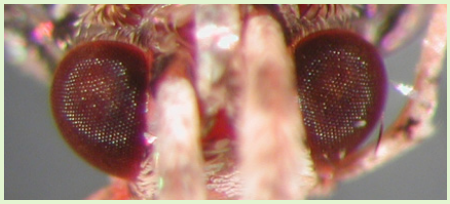


Figure 1. PBW adult with normal eye color.

Figure 2. PBW adult with mutant orange eye used as genetic marker in our study.

the number of acres a given number of sterile moths will cover, therefore reducing the numbers released per acre without impacting the effectiveness of the treatment.

Approach:

APHIS strain: 2003 Studies:

Laboratory tests were conducted to determine the F1 biopotential of the current APHIS mass-reared stain of PBW when both parents are treated with Cobalt60 at irradiation doses of 8, 10, and 12 kr. We used the standard dose of 20 kr for comparison and an untreated control. From the results of the laboratory studies we selected a dose of 10 kr for field-testing. Commercial Bt cotton fields with infield refugia were used in our field study. Fields receiving aerially released moths irradiated with 10 kr were isolated by at least 1/2 mile from fields receiving 20 kr moths. A total of ca 414,000 moths per irradiation treatment were released. Moths receiving the 10 kr treatment carried a genetic marker (orange eye, acquired through conventional nonmolecular means), while those irradiated with 20 kr had normal eye color, and all release moths were internally marked with a red dye. Since the 10kr moth carried a recessive genetic marker, any F1 progeny produced by a self cross mating of the 10 kr release population could be readily identified by their eye color and the absence of the internal dye marker. Following the first release, we used a PBW heat unit model to project the emergence of any adult F1 progeny from our release population. During the window we anticipated eclosion of adult F1 progeny, trap inspections were increased to 5 times a week. Random boll samples of 100 bolls per field were coordinated with model projections on the presence of 3rd instar larvae in the field.

2004 Studies: Similar field studies were conducted in 2004. Changes in the test protocol included ground releases instead of aerial releases of PBW moths and the cotton fields selected for the test were planted with Pima cotton, a non-Bt variety. Total moth releases were also lower with 173,141 orange eye PBW moths irradiated with 10 kr and 224,469 normal eye moths released irradiated with 20 kr, respectively.

Transgenic EGFP-0C PBW strain: In 2003 and 2004 studies were conducted with EGFP-0C PBW females (a dominant genetic marker

PBW strain) designed to measure the impact an irradiation dose has on the calling behavior (male solicitation) of female moths. We compared male solicitation ability of EGFP-0C females irradiated with 8, 10 and 20 Kr doses of Cobalt60 and APHIS strain females irradiated at 0 and 20 Kr. Single irradiated females were placed in screen cages along with a small cotton leaf. The screen cages were then placed in a delta trap. The traps with females intact were placed at dusk on the periphery of a cotton field containing a high native PBW population and were collected the following morning and returned to the laboratory for evaluation.

Accomplishments:

APHIS strain: Field test results in 2003 showed recapture rates of moths irradiated with the 10 kr treatment were 50% higher than moths irradiated with 20kr. Furthermore, no F1 moths were captured in the intensive trapping schedule coordinated in time to detect eclosion of potential F1 adult progeny produced by self-cross mating of the 10 kr release population. Boll samples also resulted in no detection of larvae carrying the genetic marker. The native PBW population in our test area exploded ca. 2 weeks following our initial releases. During our releases the sterile to native ratio was 7.8:1. However, at the time we projected adult F1 progeny in the field from release moths, the traps were overloading on a nightly basis with a sterile to native ratio of 47:1. The high number of native females in the field reduced our chances of capturing F1 by increasing competition to the traps.

In 2004, males irradiated with 10kr showed recapture rates 25.8% higher than males irradiated with 20 kr of cobalt 60. We also captured two F1 male moths in our season-long trapping survey of the test fields. However, we did not find any F1 larvae in our weekly boll samples.

The data indicate that moth quality is improved by a lower dose of irradiation based on male response to survey traps, a historical indicator of male moth quality in the field. Furthermore, under the conditions of our studies, the F1 biopotential of a release population irradiated with 10 kr of cobalt 60 would not be factor of concern in a SIT program where existing detectable native populations exist. The use of a release strain of PBW carrying a dominant genetic marker would certainly be an advantage and enhancement to an SIT program. It would open the door for the use of a lower dose of irradiation in the San Joaquin PBW SIT program, which is viewed as a preventive program, i.e. a program where no established PBW populations reside but the threat of establishment is ever present from the southern desert valleys of California.

Transgenic EGFP-0C PBW strain:

Test results indicate that young irradiated females, regardless of irradiation dose or stain type, were as successful as young non-irradiated females of the APHIS strain (control) in soliciting wild males from a field population. However, generally older irradiated females (age 6 or 10 days old) regardless of strain type were not as successful in soliciting males from a field population as were non-irradiated APHIS females. With the exception of the 2004 test 6-day-old female results, irradiated females from the EGFP-OC strain were statistically as effective as irradiated APHIS strain females in solicitation of males to a trap, regardless of the dose of irradiation they received.

Project Deliverables

- Lowering the dose of irradiation from 20 kr to 10 kr would improve the quality and performance of male moths used in the PBW SIT Program.
- Transgenic EGFP female moths irradiated at doses of 8, 10, or 20 kr were as capable as females from APHIS strain (non-transgenic strain) irradiated with 20 kr in attracting PBW males from a field population.

Pink Bollworm (PBW): Enhanced Green Florescent Protein (EGFP) colony improvement and studies to determine its fitness in confined field cages when compared to non-transgenic mass reared PBW

Ernie Miller

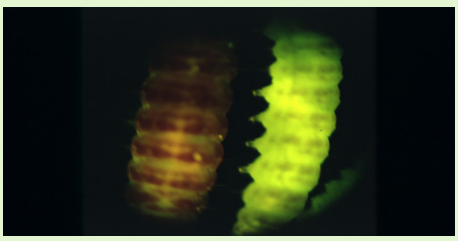
Decision Support & Pest Management Systems Laboratory, Phoenix, AZ

PROJECT HIGHLIGHTS

The EGFP strain of PBW containing a transgenic dominant marker gene is suitable for use as a test release insect in an F1 sterility program. This is based on the stability of the gene and the field performance of the strain which was measured over 3 years of confined field studies.

PROBLEM STATEMENT: Several modifications to the current PBW SIT program and/or a projected expansion of the program have been proposed that would utilize transgenic PBW. Two strategies have been suggested that would improve the overall efficiency of an SIT program. One is the use of a transgenic dominant marker system to label both the release insects and their F1 progeny in an F1 sterility program. This approach would reduce the irradiation dose applied to the release moths therefore improving their competitive fitness. A second approach is the release of a transgenic moth strain with a conditional lethal gene whose function is similar to sterility. Here the sterility of the release population is based on the transmission of a lethal gene to the field population of PBW causing lethality of F1 progeny of all probable matings with the release insects (release by wild type, or release by release). Ideally embryo-specific lethality would be the optimum. This approach would eliminate the need for irradiation, therefore improving the competitive fitness of the release insects.

APPROACH: Current studies were directed at evaluating a transgenic dominant marker system (EGFP) in a PBW strain that would label both the release insects and their F1 progeny in an F1 sterility program. Tests were designed to simulate how the laboratory reared EGFP PBW



Fourth instar larvae of normal PBW larvae (left) and EGFP larvae (right) expressing the EGFP protein

moth performed in the field when compared to its "wild type" counterpart, the APHIS strain of PBW, as would be the case for its use in an SIT program against an endemic PBW population.

The results of a confined field test in 2002 revealed that there was a competitive fitness loss in our transgenic strain of PBW. One of the likely causes of fitness loss was inbreeding depression of the strain. Therefore inbreeding depression was addressed in our 2003 studies. We out-bred the EGFP strain to both APHIS moths and a newly domesticated PBW strain collected from cotton bolls and selected to a heterozygote line for our 2003 study.

Field cages where all confined field studies were conducted with the EGFP strains of PBW

In 2004 we again out-bred the strain to a recently domesticated strain of PBW and also to the existing strain used in the PBWRF. The strain was then reselected for homozygous EGFP. We then planned to apply for a permit for an unconfined release of the strain. The unconfined release would have been incorporated into the PBW SIT/Eradication program on a pilot test basis. The first step in this endeavor after acquiring the permit was to transfer the strain into the PBWRF and mass rear it as a second strain in the facility. These objectives were not carried out due to lack of funding. Therefore, we reverted back to less costly small scale confined field testing. We conducted new tests and some tests similar to those conducted in 2003, except we evaluated the transgenic strain at a lower irradiation dose to determine if we could improve the strain's vitality and obtain similar or improved control effects on a simulated wild PBW population compared to the current APHIS strain irradiated at the fully sterilizing dose of 20 kr. This strain is the one currently being used in the PBW-SIT Program.

ACCOMPLISHMENTS: In 2002 confined field test results evaluating the competitive fitness of a EGFP strain of PBW indicated a significant loss in competitive fitness when compared to our current APHIS strain of PBW. This made it a poor candidate for implementation into an SIT program. However an out-bred transgenic strain, EGFP-0C, tested in 2003, showed some improvement in competitive fitness, though this strain also demonstrated some fitness loss in what appears to be a shorter effective field life for males than the non-transgenic APHIS strain males.

In 2004, following a second year of out breeding and reducing the irradiation dose to 10 kr, we finally have a strain that test results indicate is statistically comparable to the APHIS strain when male response to pheromone traps and or females are used as measurement of male field performance. This is a traditional method of evaluating field performance of SIT insects in the PBW SIT program. EGFP female mating ability is also not statistically different than that of APHIS strain females.

In 2004, Dr Park's laboratory at Kansas State University confirmed the stability of the EGFP transgene in our PBW strain after over 50 generations of laboratory rearing using molecular testing methods.

In 2004, confined field cage studies simulating control of a PBW population using irradiated insects indicated that EGFP-OC strain PBW irradiated at 10 kr were as effective as APHIS moths irradiated at 20kr in controlling a PBW field population.

Development of additional marker strains has been unsuccessful. We have been relying on Tom Miller's laboratory to provide these for us and though they initially reported successful development of DsRed marker strains, these later proved to be transient somatic cell transfers. Due to personnel changes and commitments to other projects, they will be unable to continue work on development of additional marker strains in the future.

Construction of the new PCR lab is now complete. We will use this lab to start our own injections with support from Greg Simmons and his staff. Our goal is to develop multiple marker strains in an effort to identify a transgenic strain of PBW with expression in the adult stage and also have a high performance level in the field.

PROJECT DELIVERABLES

- Visual screening per generation and molecular genetic analysis indicate a stable EGFP transgene in our current EGFP marker stain of PBW.
- The EGFP- Out cross (EGFP-OC) marker strain of PBW irradiated with 10 kr of cobalt-60 field performance is comparable to the current APHIS strain of PBW irradiated with a 20 kr dose of irradiation.

Development of Autocidal and Genetic Engineering Technology for Improved Pink Bollworm Sterile Insect Release Program

Gregory Simmons

Decision Support & Pest Management Systems Laboratory, Phoenix, AZ

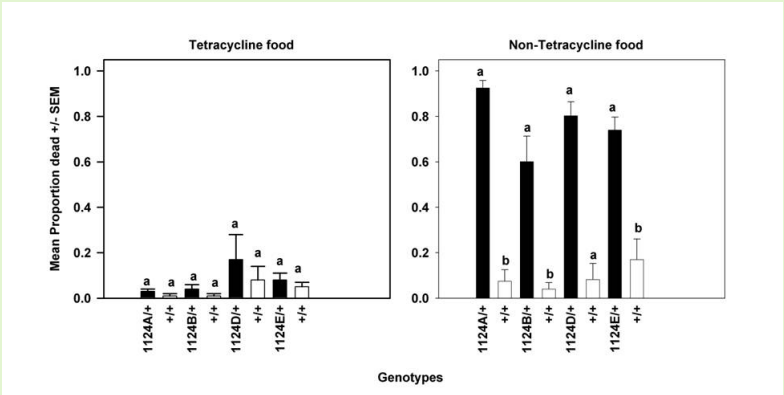
Project Highlights

Significant progress has been made in the development of pink bollworm strains genetically transformed with a conditionally lethal gene for use in a new area-wide genetic control strategy. Mortality rates as high as 92% for several transgenic pink bollworm strains were recently demonstrated in a series of laboratory rearing experiments. Introduction of a pink bollworm strain with a conditionally lethal gene into the eradication program would dramatically improve control efficacy and substantially reduce program costs. This new technology may also be transferable to other important program pests, and implementation into the pink bollworm program will facilitate development of this technology.

Pink bollworm (PBW) infestations cost U.S. cotton producers $32 million per year for direct losses and control measures. APHIS is involved in two PBW control projects using the release of sterile (SIT) PBW, PBW *Suppression* in the Central Valley of California, and PBW *Eradication* using B.t. cotton, pheromones, and pesticides. The use of SIT will expand to 90,000 acres in 2005 when sterile releases are made in eradication program areas in Texas, New Mexico and the Juarez valley in Mexico.

The SIT suppression program has been effective in keeping the San Joaquin Valley free of PBW for 30 years at low cost. However, increased cotton production costs, worldwide competition and the increasing demands for the expanded PBW eradication program requires a more effective and lower cost program. A major limit on the efficacy of SIT as a control measure is the effect of sterilizing radiation on insect performance. It is estimated that sterilized PBW have a 50% reduction in mating effectiveness compared to unsterilized moths.

The use of genetic engineering to improve PBW control technology can achieve savings and greater program efficacy in two ways: 1) By creating a genetically marked PBW strain, which could allow reducing the radiation dose by as much as 50%; 2) By the development of a PBW strain with a conditionally lethal gene, which would eliminate the need for sterilizing released insects with radiation and greatly improve the performance and longevity of released moths.



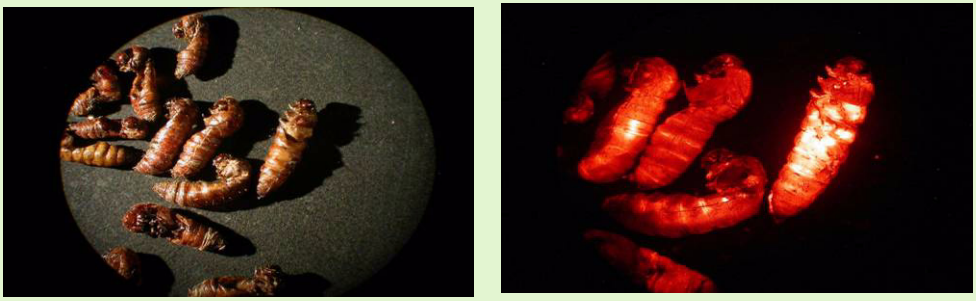
Mortality of progeny from LA1124 heterozygote males crossed with wild type females reared with and without tetracycline food. Mortality is scored at pupal and adult eclosion stages, and summed together for both stages. Each pair of bars consists of a paired experiment where the mortality of transgenic and wild type progeny reared on the same diet type are compared. Mean values for pairs of bars marked with a different letter are significantly different at $P < 0.05$.

The goal of this project is to develop a pink bollworm with a conditionally lethal gene using RIDL technology (Release of Insects with a Dominant Lethal gene) to make use of an innovative genetic control technique known as Autocidal Biological Control. Progeny carrying a RIDL gene die when the antibiotic chlortetracycline (CTC) is absent from their diet. CTC is a normal component in PBW mass-rearing so a PBW strain with conditionally lethality controlled by CTC is a good choice for this rearing system. This is one of the most promising autocidal control systems in development.

Introduction of a PBW strain with either a genetic marker or with a RIDL gene into control programs would dramatically improve control efficacy and substantially reduce program costs. The RIDL technology may also be transferable to other important plant pests, and implementation in the PBW program will facilitate its development for other pest control programs.

CPHST has formed a cooperative research project with three universities: UC Riverside (UCR), Kansas State (KSU) and Oxford University (OU); and with private industry (Oxitec, Ltd) for development of RIDL and genetic engineering technology for PBW. Work by all members of this team is summarized here.

DEVELOPMENT OF RIDL PINK BOLLWORM: During the past year, genetically transformed PBW with RIDL system constructs have been produced at the three laboratories making injections of pink bollworm. To date, twenty transgenic PBW strains with RIDL constructs have been tested at Phoenix, UCR and Oxford/Oxitec. Of these lines tested, five lines (LA1124) die when reared on CTC free diets. This construct was made with a lethal gene called tetO-tTA, which in the absence of CTC causes lethality by high expression of a lethal protein.



Dead 1124/+ pink bollworm prepupae reared on non-tetracycline food shown with and without excitation light. Left, appearance under normal light, right same insects with excitation light showing marker showing the fluorescent phenotype of the DsRed transgenic marker of LA1124/+ insects.

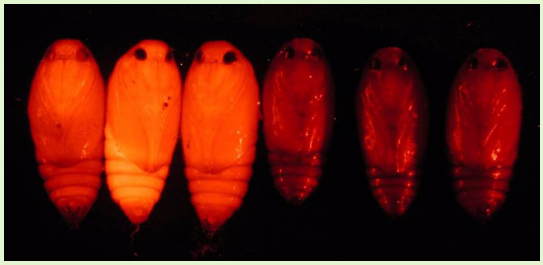
Eight rearing experiments have been conducted crossing LA1124 lines to wild type PBW to produce progeny with a 1:1 ratio of two genotypes, one with the RIDL gene, and one that is wild type. These are reared with and without CTC and mortality is measured. This experiment is designed to simulate the mortality of progeny from the mating of a RIDL moth with wild type PBW after release of RIDL moths in a cotton field.

To date, over 4,000 individuals have been tested and significant levels of mortality were shown for RIDL progeny reared on CTC free diet. Most of the mortality occurs in the prepupal stage with mean levels of mortality ranging from 60 to 92%. Rates of mortality in the control treatments (RIDL and wild type progeny reared on CTC diet, and wild type PBW progeny reared on CTC free diet) were low, ranging from 2-15%.

Other work underway includes testing adult longevity of LA1124 lines (important for both rearing and field release) and testing the rates of mortality when LA1124 PBW are crossed to transgenic lines with other lethal genes (e.g., Nipper in LA476, and cycline-B in lines LA1062 and LA1064). Here the strategy is to combine the lethal effects of two separate lethal genes in a single PBW strain to learn if mortality rates can be increased or if lethality will occur at earlier larval stages than what occurs for the LA1124 line alone.

Factory Production of Test Diets:

Having a uniform diet in sufficient quantities for testing RIDL strain lethality has been one of the largest challenges to making progress on this project. Previously, test diets with and without CTC were made by hand in small batches. Testing was limited by the small amounts of diet that could be made by this method and by problems with maintaining batch uniformity.



Healthy transgenic and wild type (APHIS strain) PBW pupae when reared with tetracycline (CTC) food as seen under excitation light. Left, LA1124/+ showing the fluorescent phenotype of the DsRed transgenic marker. Right, non-fluorescing +/+ pupae.

Recently, problems with diet quality and quantity have been solved with the recent adoption of a new CTC delivery system into the twin screw extruder (TSE) used to make the factory diet for the Pink Bollworm Facility. The new system allows the separate introduction of CTC into the diet so that TSE diet can be made without CTC whenever it is needed for testing. This is an enormous improvement over our previous method of making small batches of test diet by hand, allowing the production of many kilos of uniform high quality diet. Thanks to the hard work and cooperation of Joe Ploski and Rob Anderson of the APHIS-PPQ Western Region PBW Rearing Facility, Renee Bagneris and Robert Smith of the CPHST Gulfport Analysis Laboratory, and Ernie Miller of CPHST-Phoenix this goal was achieved.

RISK ASSESSMENT OF GENETICALLY ENGINEERED PINK BOLLWORM: During the past year, continuing progress on risk assessment of genetically modified PBW has been accomplished by CPHST personnel and by our collaborator. Dr. Yoonseong Park of KSU. Phoenix projects include testing the stability, fitness, and performance of transformed PBW under mass-rearing and field cage conditions (see reports by E. Miller) and the collection of more than 3,000 PBW specimens from 42 sites in three southwestern U.S states, Mexico, Australia and India for genetic analysis to determine the risks of using program insects transformed with the *piggyBac* transposon.

Dr. Park's laboratory analysis of wild PBW populations has shown that transposons similar to *piggyBac* are present in many of the populations surveyed. While this information suggests a potential risk factor that must be considered in the risk assessment process, preliminary genetic analysis shows that *piggyBac*-like transposons have had no or extremely low rates of movement within and between populations. This suggests that using a non-autonomous *piggyBac* transposon to create transgenic PBW strains will pose little risk to the environment. In 2005, these studies will be expanded to increase sample size and to include more populations.

Project Deliverables

- 20 transgenic pink bollworm strains produced and tested during the past year.
- First demonstration of a working autocidal/conditionally lethal gene in a genetically engineered lepidopteran pest.
- Mortality rates as high as 92% demonstrated in a transgenic pink bollworm.
- New system for producing high quality factory-made test diets implemented.
- Genetic analysis of several pink bollworm populations completed as part of risk assessment activity.

Implementation of Real-Time PCR for rapid on-site detection of citrus canker

Vessela A Mavrodieva

National Plant Germplasm and Biotechnology Laboratory, Beltsville, MD

PROJECT HIGHLIGHTS

- New improved methods for fast sample preparation that requires minimal handling were optimized.
- A SYBR Green real-time PCR has been optimized. The paper "Improved sampling methods for real-time PCR Diagnosis of citrus canker from field samples" was published in 2004 in Phytopathology, 94, pp. 61-68.
- The technology transfer of the SYBR-Green method to FDACS-DPI was accomplished in August 2003 in response to court order for molecular confirmation of the citrus canker infected trees slated for destruction.
- CDFA received a protocol for conventional PCR using universal primers developed by our lab. They also showed interest in the real-time PCR protocol.
- A Real-Time PCR Taqman assay using a universal probe was developed and tested on both R.A.P.I.D. and Smart Cycler instruments.

THE PROBLEM: Citrus canker disease is caused by two phylogenetically distinct groups of Gram-negative bacteria in the genus *Xanthomonas*. The first phylogenetic group is the Asiatic group, named *Xanthomonas citri* pv citri (Xac A, A* and AW). The second group is the South American group, named *X. campestris pv aurantifolii* Gabriel (Xac B and C). Groups of citrus canker strains differ mainly in their host range while symptoms of the disease are identical on the susceptible hosts. As a result of the disease trees weaken and fruit production drops. Citrus canker is an object of quarantine regulations, thus canker outbreaks strongly affect the domestic and international movement of citrus. The presence of the canker in Florida threatens various aspects of the $9 billion dollar industry.

Prior to August 2003, citrus canker detection was done visually by State inspectors and pathologists based solely on typical symptoms of hyperplasia (lesion) on the leaves, fruit, and stems of infected citrus. There was an obvious need for a highly specific PCR assay with primers able to detect all canker causing strains. To increase the speed of diagnosis, the assay also needed to be portable to provide rapid on-site diagnostics, with minimal handling to prevent possible cross-contamination. Previous PCR detection protocols involved several steps, required DNA extraction or antibody capture and did not work with all known citrus canker strains.

THE APPROACH: For this work we have been using a real-time PCR system from Idaho Technology named R.A.P.I.D. that provides fast and reliable detection in a portable, ruggedized system format.

Primers designed by our collaborator, Prof. Dean Gabriel at the University of Florida, and utilized in this work were based on the *pth*A gene conserved sequence. The *pht*A gene has been identified as essential for *X. citri* pathogenicity and homologous genes have been found in every *Xanthomonas* strain that causes citrus canker. This gene is not found in other plant pathogenic bacteria or fungi.

Primers were tested for their specificity with DNA from all canker causing strains and non-specific bacteria, particularly *X. campestris pv citrumelo* (CBS), and strains exhibiting a wide geographic distribution of canker. The best pair of primers were selected and used in a real-time PCR assay with SYBR Green as a fluorescence dye initially on the R.A.P.I.D. instrument. The assay was then transferred to the Smart Cycler instrument from Cepheid, because many State and NPDN labs have the Smart Cycler. Primers able to differentiate between Xac A and Xac AW have been designed and tested for their specificity. Taqman universal probe and strain specific probes have been designed and tested for citrus canker detection since the Taqman approach had been reported to achieve greater specificity and sensitivity than SYBR Green-based real-time detection.

Sampling proves to be the critical point in the adaptation of every detection assay for use on field samples or in the field directly. Different sampling procedures and preparation methods have been tested using greenhouse inoculated leaves, leaf and fruit field samples tested in the quarantine facility of FDACS in Gainesville, FL. Two major approaches have been used: a crude sample preparation (crushed lesions) and fast DNA extraction using the IT 1-2-3 RAPID DNA purification swab kit from Idaho Technology.

2004 ACCOMPLISHMENTS: More than 70% of the project's objectives have been accomplished up to date.

We designed and optimized a highly specific universal Taqman assay for citrus canker that is even faster than the SYBR Green assay. Both assays (SYBR Green and Taqman) were transferred to the Smart Cycler.

A conventional PCR using the same universal pair of primers for citrus canker detection was optimized and provided to CDFA lab. Furthermore, we provide them with the protocols of our real-time PCR assays.

New primers for discrimination of the Xac AW were designed in the lab of our collaborator Prof. D. Gabriel from UF in Gainesville, FL to meet the specific quarantine regulation for that strain. We are currently working on testing them.

COLLABORATORS: We would like to give due credit to out collaborators. All primers used for citrus canker

detection were designed in Prof. Dean Gabriel's lab from UF in Gainesville. The initial work on sampling methods and SYBR Green assay optimization were completed by our staff but in Prof. Dean Gabriel's lab in UF and using the citrus canker quarantine facility of the FDOACS - DPI. CCEP PPQ staff provided us with necessary field samples for the optimization work. We would also like to thank Tim Riley, PPQ CCEP, for his collection of field samples and swabbing of infected materials throughout this program.

PROJECT DELIVERABLES

- New improved sampling methods that require less handling were proposed.
- A robust universal real-time PCR test was optimized for rapid, reliable and on-site detection of citrus canker.
- Some of the stakeholders of FDOACS - DPI received the test and were trained in our center in August 2003.

Adaptation of plum pox potyvirus IC-RT-PCR to a real-time PCR format and use for in-field testing

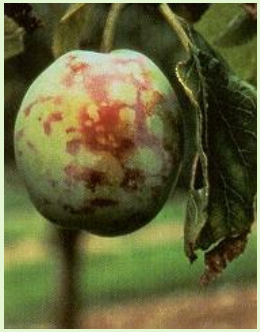
Vessela A Mavrodieva

National Plant Germplasm and Biotechnology Laboratory, Beltsville, MD

PROJECT HIGHLIGHTS

- A SYBR Green real-time IC-RT-PCR protocol was optimized using the 3'NCR universal primer pair.
- Taqman probes were designed to 2 different genome regions and corresponding assays optimized.
- The 3'NCR SYBR Green and Taqman assays were tested with numerous PPV strains and isolates, and showed good specificity. All three assays have comparable sensitivity.
- Results were presented at the 19th International symposium of virus and virus-like diseases of fruit trees in Valencia, Spain in 2003.

THE PROBLEM: Plum pox potyvirus (PPV) is a plant regulatory pathogen and a select agent. The virus was detected in PA in 1999. After virus detection in PA, a strict quarantine was established followed by an emergency eradication program that is now into its sixth year. Those measures drastically changed the face of the PA Prunus industry. Successful eradication requires, first and foremost, rapid and reliable diagnostics. Rapid and reliable PPV detection is obvious not only for the plum pox emergency program, currently being conducted by PPQ, but for nursery certification programs and for rapid response capabilities by PPQ or the NPDN.



Many different methods have been developed for PPV laboratory identification and differentiation. Of these, Real-time PCR has become widely used in molecular diagnostics due to its sensitivity, speed and availability to be deployed in different formats. Portable real-time PCR machines allow fast on-site detection of plant pests in quarantine zones, surveys plots, mobile diagnostic laboratories, and ports of entry.

THE APPROACH: To streamline the diagnostic process for PPV, we proposed a project of adaptation of our existing conventional format immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) for PPV detection protocol to a real-time IC-RT-PCR format. Our lab has been using a pair of primers based on the 3'-non-coding region (NCR) that is a conserved region of the virus RNA for PPV detection. This pair of primers has been excessively tested, by this laboratory as well as by several labs internationally using many

different PPV strains and isolates and has in all cases been found to universally detect PPV. Our efforts have adapted this assay to a real-time RT-PCR format with SYBR Green. In addition, we have developed Taqman probes for 2 different genomic regions of PPV for added specificity. We are also making a comparative evaluation of the newly developed Taqman assay with other existing Real-time PCR assays. These probes were tested for specificity. We adapted the assays for 2 different real-time PCR instruments: the R.A.P.I.D. instrument from Idaho technology and the Smart Cycler from Cepheid. To further facilitate sample processing, we have been evaluating the usefulness of an internal control for validation of the PCR results.

2004 ACCOMPLISHMENTS: Almost all of the objectives have been successfully accomplished. We transferred the SYBR Green and Taqman assays to the Cepheid instrument (Smart Cycler). Some commercial real-time RT-PCR kits were tested but none of them provided higher sensitivity than our in-house developed protocol. Sequences of the internal control amplicon were obtained from several different host plans. Currently we are working on designing of a Taqman probe for the internal control to be combined with the real-time assays as a multiplex reaction to safeguard against false-negative results.

PROJECT DELIVERABLES

- Optimized rapid and reliable real-time PCR diagnostics for PPV.
- Assays that can be performed on-site (in-field) on portable real-time PCR machines.

Implement a Rapid Molecular Identification Technology for Insects, with Major Focus on Immature Fruit Fly Species Intercepted at Ports of Entry

Don C. Vacek

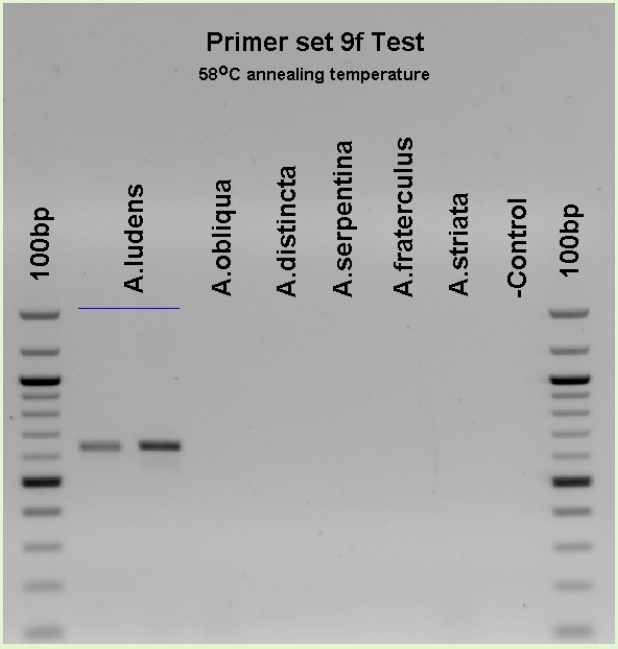
Pest Detection, Diagnostics & Management Laboratory, Edinburg, TX

PROJECT HIGHLIGHTS

A new fingerprinting method that reveals amplified, simple sequence DNA repeats (microsatellite DNA) has been established in our laboratory and can be exploited for the identification of more DNA fragments that are specific to each Anastrepha species intercepted as immature forms at ports of entry. A sequence characterized amplified region (SCAR) for identifying Anastrepha ludens, Mexican fruit fly, has been developed by Roxanne Garza, and shows promise as a molecular diagnostic. We are grateful to the Identifiers and support personnel in APHIS who have shipped us intercepted immature Anastrepha spp. from ports of entry, the technicians and scientists at ARS, Weslaco who have provided genetic fingerprinting expertise, the faithful technical and professional staff at PDDML who have provided real wet lab skills, and the CPHST headquarters staff who have provided leadership and funding. Unique DNA fragments from multiple regions of the genome of each species will insure the validity of rapid molecular diagnostics for species identification.

PROBLEM STATEMENT: APHIS does not have an effective method to identify immature *Anastrepha* to the species level. The morphological key requires late instar larvae. This key is tedious and laborious for AQI identifiers at ports of entry and at the National Identification Lab. In practice, identification stops at the genus level because the Identifier's available time is more effectively spent with the large volume of other urgent interceptions that are more efficiently identified. As a result, valuable species data intrinsic to the intercepted material are not available for risk analysis and identification of high-risk pathways of specific pest fruit flies.

APPROACH: Multiple molecular diagnostic protocols will be developed and verified for confirmation of each diagnostic determination. From selected fingerprinting methods, sequence characterized amplified regions (SCARs) unique to each species of interest will be identified. Real-time PCR with primers developed specifically for these unique SCARs will be developed and verified. This technology will be adapted to high throughput real-time PCR instruments that require a minimum of technical skills. Finally, the simplified and rapid molecular diagnostics methods will be implemented at selected ports of entry.



Agarose gel image showing the 640 base pair, 9f SCAR fragment in *A. ludens.*

ACCOMPLISHMENTS: A new fingerprinting method that reveals amplified, simple sequence DNA repeats (microsatellite DNA) has been established in our laboratory and can be exploited for the identification of more DNA fragments that are specific to each *Anastrepha* species intercepted as immature forms at ports of entry. Unique DNA fragments from multiple regions of the genome of each species will insure the validity of rapid, molecular diagnostics for species identification.

The technology for developing genetic fingerprints of inter-simple sequence repeats (ISSR) has been transferred to the Genetic Diagnostic unit at PDDML. Re-optimization of ISSR-PCR reactions and thermal cycler programs at PDDML has been necessary for adapting the procedures learned from Dr. Jesse DeLeon, ARS, Weslaco. Additional individuals from several populations of six *Anastrepha* species of economic importance have been assayed for ISSRs with specific primers. ISSR banding patterns generated at PDDML continue to show species specific differences. ISSR, DNA fragments that are monomorphic for a species potentially contain unique DNA sequences for development of diagnostic markers.

A sequence characterized amplified region (SCAR) from previously identified unique RAPD-PCR fragments from *Anastrepha ludens*, Mexican fruit fly, has been developed by Roxanne Garza. The RAPD fragment was cloned and sequenced. Forward and reverse DNA primers, named 9f, were developed and verified to amplify a specific sequence (the potential SCAR) in the *A. ludens* RAPD fragment. The SCAR fragment appeared as a single, intense, 640 base pair DNA band (Fig. 1) and was amplified with primer pair 9f from all of 36 *A. ludens* individuals representing a total of 14 populations from the following countries, respectively: 9 - Mexico, 4 - Guatemala, and 1 - Honduras. Each of 5 other species of Anastrepha was represented by 4 populations from Mexico and Guatemala. The 9f SCAR fragment was not amplified from any of the 14 *A. serpentina*, 10

A. striata, and 14 *A. obliqua*. The 640 base pair fragment did appear as a faint band from 50% of the 12 *A. distincta* and from 30% of the 12 *A. fraterculus* individuals. Current data show a consistent high yield of the 9f SCAR fragment as the sole amplicon from *A. ludens*. The negative data from *A. serpentina, A. striata*, and *A. obliqua* and the low to non-detectable yield from *A. distincta* and *A. fraterculus*, support the 9f SCAR fragment as a genetic marker for *A. ludens*. The best diagnostic process would include a SCAR marker for each of the species. Unique RAPD fragments from *A. serpentina, A. obliqua*, and *A. fraterculus* have been cloned, and some have been sequenced in preparation for development of SCAR markers. Ultimately, SCARs will be developed from unique fragments identified with three fingerprinting techniques, RAPD-PCR, PCR-RFLP, and ISSR. SCAR markers will serve as a basis for developing real-time PCR diagnostic methods.

PARTNERSHIPS: Through communication bridges since 2000, Identifiers at Ports of Entry in Texas have been shipping interceptions of immature stages of *Anastrepha* species to our laboratory. At the 5th Meeting of the Working Group on Fruit Flies of the Southern Hemisphere (May 2004, Ft. Lauderdale, FL), contacts were made and renewed and an *Anastrepha* field collection protocol was developed with National Identifiers and distributed to Field, Region, IS personnel, and fruit fly researchers in the Western Hemisphere. As a result we have received materials from the Caribbean region. During the fall and winter, extensive training in ISSR-PCR for DNA fingerprinting and development of SCAR markers was received at the Beneficial Insects Research Unit of ARS, Weslaco.

PROJECT DELIVERABLES

- ISSR-PCR, microsatellite DNA fingerprinting for species specific fruit fly diagnostics
- New fingerprinting method established at PDDML
- SCAR fragment – promise as a Anastrepha ludens, Mexican fruit fly, diagnostic
- Unique DNA fragments from multiple regions insures validity of diagnostics
- Thank you: PPQ Identifiers; BIRU,ARS, Weslaco; PDDML techs; & CPHST HQ

Molecular Diagnostic Techniques That Identify Foreign Sources of Fruit Fly Pest Introductions and Procedures for Establishing the Pest Detection, Diagnostic and Management Laboratory as a Center for Fruit Fly Diagnostics

Raul A. Ruiz

Pest Detection, Diagnostics & Management Laboratory, Edinburg, TX

PROJECT HIGHLIGHTS

- Mitochondrial-based technique that distinguishes *A. ludens* from 5 other related species of economic concern was developed.
- Over 30 collections of *A. ludens* were gathered from Mexico and Central America.
- Microsatellite DNA regions show population variation increasing potential for determining the source of pest introductions.

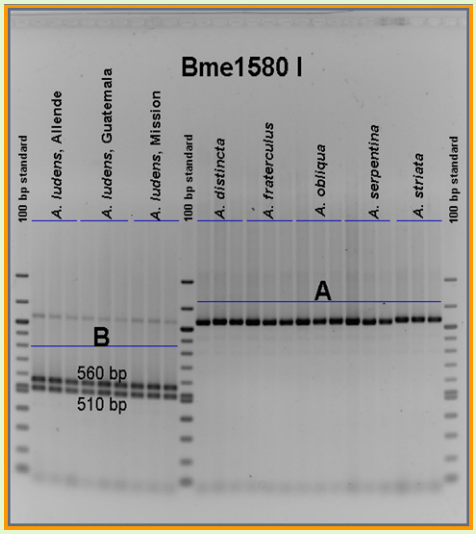
PROBLEM: Millions of dollars are spent annually on the control and eradication of fruit pest species such as *Anastrepha ludens*. Currently, there are no known methods for accurately determining the source of fruit fly pest outbreaks such as the Mexican fruit fly in the U.S. Traditional methodologies currently used by PPQ identifiers and taxonomists are not effective for identifying the source of infestations. Determining the source of intercepted Mexican fruit flies will serve as valuable information for risk assessment and pathway analyses where millions of dollars in agriculture trade are affected.

APPROACH: Molecular techniques that reveal population variation commonly use mitochondrial DNA as well as other DNAs for studying evolutionary relationships. These powerful genetic tools are used to infer evolutionary relationships by incorporating technologies such as PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism). Known fruit fly chromosomal and extra-chromosomal genome sequences provide the necessary raw material for comparing gene arrangements. These sequence variations, previously used in determining the origins of fruit fly pests such as Mediterranean fruit fly (*C. capitata*), are being studied here.

ACCOMPLISHMENTS: Collaborative efforts with USDA IS and USDA ARS federal agencies yielded over 30 collections of specimens from Mexico and Central

America in 2004. Approximately 1,500 larvae and adults were gathered and subsets were used in testing the above mentioned techniques. The results produce species-specific banding patterns for *A. ludens* and provide a mitochondrial-based molecular method for distinguishing this pest species from other related species regardless of life stage (Fig. 1). In the process of developing the methodologies, various mitochondrial fragments from *A. ludens* and 5 other related *Anastrepha* species were cloned and sequenced. The sequence information is being used to develop species-specific assays that will distinguish *A. ludens* from other related species using conventional and realtime PCR for technology transfer. We have successfully sequenced mitochondrial coding regions and are currently in the process of developing these species-specific probes. Also, the various fragments sequenced from the above mentioned species will be submitted to Genebank and referenced in a manuscript currently under review.

As a result of networking with other fruit fly molecular biologists at the 5th Meeting of the Fruit Flies of the Western Hemisphere, microsatellite sequence information was obtained and PCR methodologies were tested on recently recovered (by Don Thomas, ARS) specimens of *A. ludens* from various Mexican states.

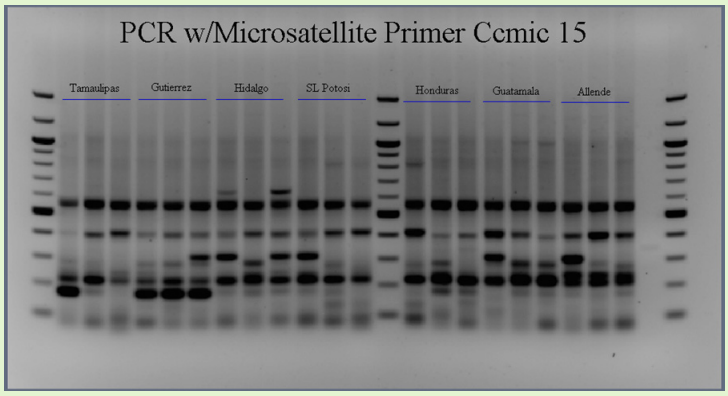


Haplotypes A and B produced from the PCR-RFLP of mitochondrial DNA showing unique banding for the Mexican fruit fly (*Anastrepha ludens*).

A total of 10 primers were used to screen 3 specimens from each of the 7 geographic populations. Five of the 10 primers investigated show potential for distinguishing the 7 populations tested (Fig 2). Further testing is underway to evaluate this technique on a larger sample size.

As a result of interaction with Jesus Deleon, ARS, Weslaco, ISSR-PCR (Inter-sequence sequence repeat PCR) methods previously used for distinguishing glassy-winged sharpshooter adults were tested on *A. ludens* populations. These assays were performed on the Cepheid Smartcycler. Preliminary results show variation between populations but will require further study.

PARTNERS: Bruce A. McPheron, Pennsylvania State University, State College, PA., Martha Heath, USDA ARS, Miami, FL., Donald Thomas and David C. Robacker, USDA ARS Weslaco, TX , Pedro Rendon USDA APHIS IS Guatemala City, Guatemala, and A. J. Martinez USDA APHIS CPHST Edinburg, TX.



Products from amplification with microsatellite primers provided by M. Bonizzoni, Univ of Pavia. Variation in banding for the above populations merits further investigation.

Development of molecular techniques that identify cryptic thrip species

Raul A. Ruiz

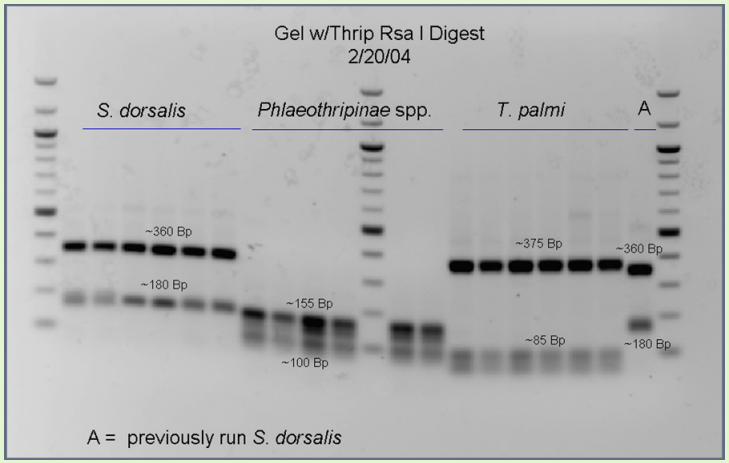
Pest Detection, Diagnostics & Management Laboratory, Edinburg, TX

PROJECT HIGHLIGHTS

- Using PCR of ribosomal DNA and RFLP techniques allows for the accurate identification of once difficult to identify thrip species that including *Scirtothrips dorsalis*.
- Collaboration with foreign agencies has been established within this new project and as a result has yielded various collections of thrip species with more to arrive soon.

PROBLEM: .*Scirtothrips dorsalis* is considered widespread throughout much of Asia and is present to a lesser extent in Africa, Oceania and the Hawaiian Islands. In general, this region has a climate that varies from temperate to tropical. The currently reported global distribution of *S. dorsalis* suggests that the pest may be more closely associated with biomes characterized as temperate broadleaf and mixed forests, tropical and subtropical dry broadleaf forests (not in the U.S.), and tropical and subtropical moist broadleaf forests. Venette & Davis (2003), based on biome matching analysis, have determined that about 28% of the continental U.S. would have a suitable climate for this thrip. Their analyses, however, does not account for conditions typical to disturbed agricultural systems and does not attempt to identify the potential for establishment and impacts on agricultural production. The risk to agricultural production posed by the introduction of *S. dorsalis* into the US is still undetermined at this time. This pest is highly polyphagous and is considered to be a significant pest of chili pepper, citrus, castor, cotton, onion and other crops in tropical and subtropical regions of Asia, Africa, Eastern Europe, Oceania, and Japan (Ananthakrishnan 1993, CABI/EPPO 1997, CABI 2003)

APPROACH: The molecular techniques proposed for this study will use ribosomal and mitochondrial DNAs for showing phylogenetic and evolutionary relationships. Ribosomal and mitochondrial DNA-based restriction fragment polymorphisms have proven useful by Toda and Komazaki (2002) in determining the identity of cryptic thrip pest species such as *S. dorsalis, Thrips palmi, Thrips tabaci, Frankiniella occidentalis*, and others, and have been of great use in determining the identity and origin of other arthropods. Techniques that provide the identity and origin of infestations of the Mediterranean fruit fly (Gasparich et al., 1995; McPheron et al., 1994; Shepard et al., 1992; Steck et al.,



Restriction digest products from amplified ribosomal DNA technique show mobilities at PDDML duplicate Toda and Komazaki (2002) results.

1996) are in place at the PDDML and have been widely used in studies of introduced insect populations such as *Anastrepha fraterculus* and other fruit fly species (Heath et al., 2001). These molecular tools are used to distinguish at the species-level and infer evolutionary relationships by utilizing technologies such as PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism). Known thrip chromosomal and extra-chromosomal genome sequences have also provided the necessary raw material for comparing gene arrangements (Brunner et al, 2002). These sequence variations and others previously used in determining the origins of pests will be further studied here.

ACCOMPLISHMENTS: The molecular techniques developed by Toda and Komazaki were successfully tested at the PDDML during 2004. The expected amplicons were observed for *S. dorsalis* and *Thrips palmi* according to Toda and Komazaki's (2002) data. The digestion of these amplicons also revealed the expected banding patterns and distinguishing between the similar taxa is now facilitated (Fig. 1). Communications with foreign government and state agencies provided the PDDML with substantial leads on sample availability and have yielded samples from the West Indies, China, and Florida. Samples from Australia, Taiwan, and Israel will be arriving soon to supplement our existing collection.

PARTNERS: Ministry of Agriculture, Nature and Food Quality. Plant Protection Service, The Netherlands; Central Science Laboratory, York, UK; USDA ARS Australian Biological Control Lab; USDA ARS WCRL; Univ. of Florida, IFAS; USDA APHIS IS; USDA APHIS PPQ Eastern Region.

Development of methods for early detection of *Ralstonia solanacearum*, R3B2 in water effluent

Dawna Jones

National Plant Germplasm and Biotechnology Laboratory, Beltsville, MD

Project Highlights

Steps have been taken this year to develop an accurate large scale nondestructive method to sample geranium production facilities and consistently detect Rs R3B2 from water samples. We have successfully used the Agdia Immunostrip as a real-time PCR template for confirmation of Rs in water samples. We have also successfully detected RsR3B2 from filters used to concentrate water samples in a laboratory setting.

THE PROBLEM: *Ralstonia solanacearum* Race 3 biovar 2 is a bacterial pathogen of potato that is entering the U.S., sometimes asymptomatically, on geranium cuttings from off-shore production facilities. This pathogen was also named in the Agricultural Bio-terrorism Protection Act of 2002 as a potential weapon of agricultural bio-terrorism. The pathogen is now considered a Select Agent and possession, storage, and destruction of this bacterium is strictly regulated. In 2003, APHIS placed holds on 921 greenhouses in 47 states costing the industry nearly 7 million dollars because of destruction of over 2 million plants in the 127 facilities where RS was detected in plants generated from cuttings from Kenya. During the 2003 crisis, an early detection of *R. solanacearum*-infected geraniums from another Goldsmith facility in Guatemala resulted in a ban on importation from geranium production facilities in Guatemala until the facility could prove it was free of *R. solanacearum*. There was no accurate, large-scale nondestructive method available to sample and detect the pathogen in the large setting of a greenhouse, so individual plants had to be tested prior to cutting. In 2004, additional cuttings containing *R. solanacearum* were detected in the same facility in Guatemala and nearly 20 million plants went unsold from this one producer. Since APHIS could not determine if all infected plants were found, the facility was prohibited from shipping cuttings for the 2004 season.

THE APPROACH: To date no accurate, large scale nondestructive method is available, and during the FY04 shipping season CPSHT scientists attempted to develop a method for use. In order to develop an accurate method, additional work needed to be performed. To that end, two trips by Dr. David Kaplan, National Science Program Leader for IPM & Eradication, to Guatemala put two teams (Raleigh and

Beltsville) into action to develop an accurate, nondestructive sampling and detection method by August 2005. The new procedure will be available for the upcoming shipping season in Guatemala and elsewhere to allow off-shore sampling and testing of geranium in production facilities. The new protocol could save the geranium industry millions in lost revenues, and APHIS millions in inspections, detection, and compensation.

Preliminary work was conducted on a water collection system in a greenhouse in Guatemala in January 2004. Water will be collected and passed through commercially available filter devices and the bacteria will be detected using a sensitive and accurate method such as PCR. For this project, detection will be accomplished using a real-time PCR developed by the Central Science Lab in the UK. Use of solid collection devices to deliver a target for PCR testing have, to date, been used mainly for detection of plant viruses. In this workplan, we will overcome the obstacles of using solid matrixes to deliver targets for PCR detection. An additional complication of detecting one particular target in an environmental sample is its detection above the background of contaminating organisms. To determine the feasibility of PCR from the solid matrices, both water collection filters and immuno-flow devices spiked with *Ralstonia solanacearum* will be used in real-time PCR to determine threshold of detection and suitability as PCR template. To determine a method to suppress competing bacteria or enhance Rs, various temperatures and incubation times will be used to determine optimal enrichment conditions for RsR3B2 from collection filters. Once all of the background work above has been completed, we intend to create a greenhouse setting in the BSL-3 facility in Beltsville using Rs-infected geraniums in a cultivation system similar to that used in Guatemala to test the water collection and detection system. Any necessary alterations will be made and the system deployed via technology transfer to off-shore facilities. In FY06, facilities testing positive for Rs through water sampling will be confirmed from water collection filters delivered to NPGBL via express delivery. The use of real-time PCR for detection should allow for data reporting in 24-48 hours after receipt, depending on the number of samples received.

2004 ACCOMPLISHMENTS: Several objectives of the initial water effluent testing project have been met successfully. We have been using the commercially available test for Rs, the Agdia Immunostrip (Agdia, Inc, Elkhart, ID) as a template source in CSL real-time PCR to accurately and consistently detect Rs, Race 3 biovar 2. Various means of processing the Immunostrip for use as template were

explored, as well as the use of different parts of the Immunostrip as template source. It was determined that Agdia Immunostrips producing a clearly visible positive test result can be subject to DNA extraction using a commercially available kit designed for forensics. This DNA can then be used in a downstream real-time PCR reaction for consistent detection of Rs Race 3 biovar 2. The threshold of detection for the Immunostrips, in our hands, has been determined to be approximately 106 CFU/ml. Since detection of the bacteria from the Immunostrips in CSL real-time PCR seems to be directly related to a positive result on the Immunostrip, this is also our threshold of detection for PCR. This method will be useful in determining false positives caused by water source or other factors inherent in field detection.

A commercially available filter apparatus was successfully used to concentrate laboratory water samples spiked with Rs cells. The filters from these units were effectively used as a template source in CSL real-time PCR for detection of Race 3 biovar 2. Various means to process the filter for DNA extraction were explored, including washing the cells from the filter, direct extraction from slices of the filter in lysis buffer and maceration of the filter by freezing in liquid nitrogen and processing in a mini bead-beater. The most successful, straightforward method was the bead-beater protocol. Using this method Rs cells can be detected at a concentration of approximately, 103 CFU/ml of water sample. Since these filters can be used to concentrate 100 ml of water, the likelihood of detection in an environmental sample is increased greatly. Testing of this newly developed protocol is currently being conducted in the BSL-3 facility in Beltsville, MD.

Several external collaborators are partnering with us in an effort to meet the industry's need for a large-scale nondestructive method of sampling for Rs Race 3 biovar 2 infections. The Central Sciences Laboratory of John Elphinstone in the UK was responsible for the development of the real-time PCR test currently being used for Rs detection. Dr. Elphinstone has also been instrumental in developing a method for enriching samples for Rs that would normally be below the threshold for detection. His lab will also be validating the protocol developed. Mike Klopmeyer of Ball Flora Plants has supplied us with the geraniums and potting medium necessary for the greenhouse portion of these experiments and Caitilyn Allen's lab at the University of Wisconsin will be testing a protocol similar to the one developed in Beltsville, Guatemala.

PROJECT DELIVERABLES

- A water collection protocol and filter collection protocol for Rs R3B2.
- A protocol for serological field detection for Rs R3B2.
- A protocol for PCR (DNA) testing of Rs R3B2 from filters pre-enrichment.

Analytical and Natural Products Chemistry Laboratory (ANPCL)

Robert D. Smith

Analytical & Natural Products Chemistry Laboratory, Gulfport, MS

INTRODUCTION: ANPCL provides a quality laboratory service in support of multiple APHIS programs and CPHST work plans. Technical support is administered through methods development and method application on samples submitted from APHIS field samplers and CPHST stakeholders. As a technical service organization ANPCL stresses both "Quality" and "Timeliness" in reporting data to all customers. In 2004 ANPCL completed multiple support activities spanning a diverse scope of work projects including:

SUPPORTING APHIS ENVIRONMENTAL ERADICATION AND SUPPRESSION PROGRAMS: "ROUTINE ANALYSIS" GENERAL LABORATORY STAFF: (ON-GOING PROGRAM SUPPORT).

As a primary and on-going work project ANPCL provides analytical and organic chemistry support to Plant Protection and Quarantine (PPQ) and other Center for Plant Health, Science and Technology (CPHST) laboratories concerning nation wide Eradication and Suppression programs. Primary stakeholders include APHIS program leaders, the Environmental Monitoring Team and CPHST Scientists.

In 2004 ANPCL completed 2118 samples as submitted from the field. An additional 641 samples were completed in related methods development and validation studies. Core programs that submit samples under the scope of "Routine Analysis" include:

ASIAN LONGHORN BEETLE ERADICATION PROGRAM: Specialized and routine analysis of multi-matrices (typically trees and leaves) treated to eradicate the Asian Longhorn Beetle. Ground water samples were also submitted for trace level (ppb) analysis to monitor

Sample Preparation Grinding, Homogenization & Sub-sample mixing, chain of Custody Unit

treatment impact on drinking water systems. Sampling is seasonal (March - October) and unpredictable.

Boll Weevil Eradication Program:

This program was developed to eradicate Boll Weevil from all cotton growing areas in the U.S. and Northern Mexico. Samples are submitted seasonally (between May - November) based on pesticide applications in the field. Sample loads vary each month and samples often require priority response (less than 5 working days to analyze and report findings). Purity and tank mixture samples are also tested to ensure the purity of the pesticide used in the program and control environmental impact.

Automated Solid Phase Extraction (SPE) applications--For sample extract Clean-up

Rangeland Grasshopper and Mormon Cricket Suppression Program:

This program was developed to control the targeted insects in several Western states. Samples are submitted seasonally (between May - October) based on pesticide applications in the field. Sample loads vary each month and samples often require priority response (less than 5 working days to analyze and report findings). Purity and tank mixture samples are also tested to ensure the purity of the pesticide used in the program and control environmental impact.

Fruit-Fly Attractants and Lures:

Specialized studies on various attractants are submitted by contractors for chemical analysis prior to supplying the active ingredients for lures and attractants. ANPCL also performs chemical analysis on lures (plugs) in conjunction with Bio-assay testing (performed by APHIS -HIFFRF). The resulting data is used to determine if contract specifications are met.

Fruit-Fly Exclusion and Detection:

This program was developed to control and eradicate fruit flies, primarily the Mediterranean fruit fly and the Mexican fruit fly. Samples are submitted for residue analysis as needed. Sample load is dependant on seasonal activity in the field and is highly variable and unpredictable.

Imported Fire Ant Quarantine: This program was developed to control the artificial spread of fire ants within the United States. ANPCL analyses samples to verify quarantine treatments (pesticide applications to soil mixes) for Nursery Stock.

METHODS REVISION AND ADAPTATION- PROJECT LEADERS: FLOWERS WHITE AND LISA MOSSER: ANPCL consistently revises existing technologies and methods to provide customized working procedures for monitoring programs. This work involves extensive use of creative techniques in order to effectively establish working methods that will enable the analysis of diverse matrices. This work is often performed after new matrices have been sampled and submitted to the laboratory and backlogs have developed. ANPCL consistently dedicates resources for the evaluation of existing methods for; (1) Streamlining, (2) Cost effectiveness, (4) Automation and, (3) Environmental stewardship (use of less hazardous chemicals and chemical waste reduction). Our goal is to develop methods that provide customers with the best available resources and to posture the laboratory in the event of an "outbreak" requiring the analysis of multitudes of samples in a short time frame.

SO HOW DID WE SUPPORT OUR APHIS CUSTOMERS IN 2004?

Overall Defined Classifications with Deliverable due Dates	Total Samples Run	Target Deliverable	Actual average Deliverable
Malathion Purity Analysis	103	**10 working days**	11.22 working days
Priority[1] Analyses (all programs)	371	**5 working days**	9.93 working days
Routine[2] Analyses (all programs)	1734	**21 working days**	12.75 working days

- Priority samples as requested on sampling forms are started as soon as they are received. Deliverable dates include all re-check analysis times.
- Routine samples as requested on sampling forms are started after priority samples when both types are received. Deliverable dates include all re-checks.

OVERVIEW:

- Malathion purity analysis took slightly longer than targeted based on the nature of the analysis (substantially less sample preparation and extract clean-up) enabling the laboratory to conduct re-checks in a shorter time frame.
- Priority work took longer than targeted due to the fact that many of these high profile samples require multiple re-checks (complete re-analysis) and on the fly methods development, coupled with instrumental adjustments. The diverse matrices and trace levels involved also caused re-analysis issues. Full re-analysis often doubles the response time.
- Routine work was completed well within expectations due to management work plans, well developed and robust methods and commitments by staff to provide the highest quality services in the shortest time frame.

WHAT ARE WE DOING TO IMPROVE OUR APHIS ERADICATION AND SUPRESSION PROGAM SERVICES?

- In late 2004 we contacted our customers and requested input on how we can better serve them.
- We continually re-invent our established methods to provide the best response to program needs.
- We are expanding our commitment to communication with our customers in order to serve them better.
- We are investigating and implementing new methods and techniques in order to protect the environment by generating less waste associated with conducting chemical analyses.
- We are utilizing automated procedures whenever possible to foster methods that require less labor and reduce exposures to hazardous materials.

ORGANICS LABORATORY PROJECTS (APHIS SUPPORT)- PROJECT(S) LEADER: DR. DAVID CAMPBELL: **A secondary Critical mission task includes specialized project work requiring independent application of state of the art technology, specialized training and techniques in support of APHIS and Homeland Security Commodity Review Programs.** Work involves specialized work units and equipment specifically allocated for the projects. Sub-projects and work plans within the organics unit include:

Synthesis of pheromones for the Rosy Russian and Siberian gypsy moths. (In-progress, estimated completion in Mid 2005) **Stereo-specific synthesis of pheromone(s) utilizing Nuclear Magnetic Resonance (NMR), High Pressure Liquid Chromatography (HPLC) preparative system, Thin Layer Chromatography (TLC) and Flash Chromatography.**

- In 2004 the unit analyzed 884 samples in support of this on-going project. The pheromones produced will be used in trapping experiments by the CPHST-Otis Laboratory to determine efficacy in monitoring infestations.
- The Siberian gypsy moth pheromone will be synthesized upon completion of the Rosy Russian pheromone synthesis. Partners include: USDA, APHIS, PPQ, CPHST, OTIS laboratory scientists, Vic Mastro and David Lance.

Identification of smuggled commodities using the elemental ratio as determined by Inductively Coupled Plasma Mass Spectrometry(ICP/MS). (In-Progress, expected to continue) **The purpose of this work is to predict the origin of a commodity utilizing existing profiles based on ICP/MS generated data on similar commodities from known origins. The procedure provides valid results by direct comparison of unknown samples to well developed and supported libraries of similar commodities.** Sample based library of elemental composition by ICP/MS for a number of commodities such as

potatoes, avocados, mangos and parvals are in the process of being completed.

- In 2004 the unit completed 3,615 samples using ICP/MS. Statistical modeling (comparison of unknown to most probable known or determined origin) is supported by an error rate of less than 2 percent for either the library approach or the direct comparison approach.

The advantage of the library approach is reduced cost once a data library is established and a higher statistical assurance that the classification is correct. Statistical techniques were also developed to identify commingling and the source of the commingled commodities. This work will enable APHIS to more effectively and objectively address commodity origin issues in support of domestic marketing and aid Homeland Security by answering questions such as "Was the Idaho Potato really from Idaho, or Nebraska, or Maryland, or even the USA? Partners include: Scientists with the Safeguarding, Intervention and Trade Compliance (SITC) regional offices.

Development of a simple field test for the determination inorganic bromide (In-progress, expected completion Mid 2005) **This test will allow field personnel to simply determine if commodities were in fact fumigated (treated) with Methyl Bromide. The method developed, evaluated and identified the necessary hardware (Selective Bromide Probe) to determine if a commodity had been treated with the fumigant Methyl bromide.** We are currently testing real world samples to demonstrate the utility of the method and the application of "Selective Probe" technology.

- In 2004 the unit evaluated 116 samples in support of this work objective.

When completed, this investigation should enable APHIS field personnel to scientifically determine if the fumigant was in fact used utilizing Selective Probe technology. Direct application by field personnel will enable improved service to APHIS customers. Partners Include: Scientists with the Safeguarding, Intervention and Trade Compliance (SITC) regional offices.

Specialized Liquid Chromatograph Mass Spectrometer (LC/MS)

Development of a method for identifying noxious weed seeds using High Pressure Liquid Chromatography. (In-progress, expected to continue through 2005) **The Unit is evaluating the exploratory work done by Glenn Freeman from the New Jersey Department of Agriculture, in order to develop a working method for APHIS applications. The exploratory work enabled the identification of several weed species utilizing protein profiles from the alcohol extraction of the seeds as determined by HPLC analysis.**

- In 2004 the unit evaluated 642 samples in support of this work objective.

Potentially, noxious weeds could be imported into the United States in other seed commodities such as oats or rice. Our initial data demonstrate that this work can be extended to other species and is not limited to just seeds. There is also evidence that it will probably be possible to distinguish between varieties of the same species. This work will provide additional tools in aiding APHIS in the identification of harmful plant species that often "hitch a ride with typical imports". Partners include: Exploratory work and research with Glenn Freeman.

Development of a field method for identifying smuggled or diseased commodities in bulk cargo using Ion Mobility Spectrometry(IMS), or Time-of-flight Mass Spectrometry and Gas Chromatography Mass Spectrometry. (In-progress, expected to continue through 2005) **This project investigated portable mass spectrometry (Ion Mobility Spectrometry (IMS) technology & methodology) to identify smuggled commodities and invasive pests by their volatile organic chemical (VOC) signature.** Increased international trade has severely compromised US agricultural security due to the propensity of foreign products to enable the transport of invasive species into the country. High-throughput screening methods are needed to detect invasive pests, or smuggled commodities that may harbor the harmful pests. It is well known that agricultural products and materials infested with insects produce chemicals that are unique and can be used as a tool to detect their presence.

- In 2004 the unit evaluated 402 samples in support of this work objective.

The end result of the investigation is to determine if this technology is viable for APHIS use in providing a highly specific, user-friendly system to detect invasive pests at points of entry. Partners include: Amy Roda, USDA, APHIS, PPQ, CPHST-Subtropical Horticulture Research Station Miami, Florida.

CPHST SPECIAL PROJECTS AND STUDIES:

ANPCL also conducts chemistry based support studies for other CPHST scientists as part of their work plans and investigations. These projects are often prefaced by a development phase, followed by sample analysis for targeted compounds as requested, within a limited time frame. Each project has an independent leader, or team with a defined outcome and unique chemical challenges.

Special CPHST Project:
The Extraction and Analysis of Dinotefuran in Leaves, Twigs and Sap
Leader: Lisa Mosser (In-progress, expected completion early 2005)

The Analytical & Natural Products Chemistry Laboratory (ANPCL) was tasked with the development of an extraction and analytical method for the detection of Dinotefuran in submitted sample matrices including; leaves, twigs, and sap. The Asian Long horned Beetle (ALB) program has been tasked to develop alternative pesticide treatments for at-risk trees in ALB infested areas. Three injection systems, the AceCap, the ArborJet VIPER, and the USDA injection unit are being evaluated by the Pest Survey Detection & Exclusion Laboratory. Because of its solubility in water and the expectation of injection ease the insecticide Dinotefuran was selected for this evaluation.

Experimental Design
Modifying existing methodology and the use of a newly acquired Liquid Chromatography Mass Spectrometer (LC/MS) enabled the experimental design selected for the project. LC/MS detection was chosen based on its powerful separation technique, its sensitive detection and identification properties utilizing difficult (dirty) matrices.

- **Accomplishments**
  In 2004 the methods development unit analyzed 466 experimental samples during the method development phase to successfully develop a viable working method for leaves utilizing LC/MS technology.
- After initial method development late in 2004, a total of 39 leaf samples were evaluated and reported with 20 samples still pending.
- In 2005 a method for twigs and sap is scheduled for development with 56 samples pending the new method.

Project Impact
Successful completion of this project impacts the agency in the following manner:

- Data generated enables the Pest Survey Detection & Exclusion Laboratory to evaluate the various injection techniques and determine

Specialized sample preparation Equipment and "Clean Rooms" for Organics laboratory projects

if sufficient residue levels of Dinotefuran can be achieved in injected trees.

- Successful use of LC/MS technology enables the use of a powerful analytical tool for the detection of various compounds at low levels. Such a technique allows the agency to monitor the impact of insecticides to the environment as well as the general public.

Partners include: Dr. Phil Lewis, CPHST - Pest Survey Detection & Exclusion Laboratory, Wildlife International, Ltd., and the California Department of Food & Agriculture Laboratory

Special CPHST Project:
The Extraction and Analysis of Chlortetracycline, Tetracycline and Oxyteracycline in Pink Boll Worm Rearing Facility Diets
Leader: Renee Bagneris (In-progress, expected completion early 2005)

ANPCL was tasked with developing a method and applying the method to submitted samples using High Pressure Liquid Chromatography (HPLC) technology to determine antibiotic content in worm rearing diets. The Rearing Facility had experienced worm deaths on a diet that had been successful for them in their rearing efforts for years. They needed to analyze samples of the diet they were preparing by hand for specific antibiotics including Chlortetracycline (CTC), Tetracycline (TC), and Oxyteracycline (OTC) to find out why the worms were dying off. ANPCL obtained the antibiotic standard material and developed an adapted extraction and HPLC instrumental method for separating and detecting the targeted compounds. Calculations based on the dry and wet ingredients were made to determine the expected content of the antibiotics of interest. Finally, through several trials, we developed a method that gave

reproducible results. Next, several aspects of the hand mixed diet were prepared and analysis requested. Samples were submitted representing various stages of diet preparation including: (1) prior to fortification with CTC, (2) the diet with CTC not dyed, (3) the diet with CTC dyed and, (4) the Dye itself. Wide variability in the CTC content in hand diets reported by ANPCL to project partners led them to try using an industrial mixer for diet preparation. The rearing facility then needed comparison data between hand mixed and mechanical mixer. To lessen variability of CTC content, timed studies were conducted of all the diets with and without, CTC and Dye several times to determine which method of preparation was best.

- In 2004 this project analyzed 383 samples in support of this CPHST project.

Partners include: Gregory Simmons at the Phoenix, AZ Pink Boll Worn Rearing Facility and Ernie Miller, AZ PPPC.

Special CPHST Project:
The Extraction and Analysis of Targeted Pyrethroid(s) from Burlap
Leader: William Guyton (project completed)

The ANPCL objective was the development of extraction and detection methods to evaluate burlap samples for pyrethroid content in support of the Asian Longhorn Beetle Eradication Program-Special Study being conducted at the CPHST -Otis Facility. The primary work plan involved field applications of burlap infused with select pesticides. The project objective was to evaluate efficacy of physical traps and adulticide bands for ALB applications in the field. A Gas Chromatograph with Electron Capture Detection (GC/ECD) analytical method was developed for analyzing the pyrethroids of interest.

- In 2004 the unit evaluated, developed the method, extracted and evaluated 80 samples and reported findings. The work was performed from June 2004 through October 2004.

The data generated included unfortified and fortified control samples. The sample extracts generated were analyzed by GE/ECD in order to determine a dissipation curve for the pesticides of interest.

- An estimated 130 injections were made due to the high concentrations of pyrethroids found in the samples submitted.

Partners included: Primary project coordinators including USDA, APHIS, PPQ, CPHST scientists Ron Mack, Vic Mastro and David R. Lewis in support of the CPHST work plan concerning the "Evergreens Cemetary, Queens, NY Burlap Residue Analysis Project."

Special CPHST Project: Determination of Imidacloprid Formulations using HPLC
Leaders: Bich Tan & Joyce James (project completed)

This Project was conducted by request of the CPHST-Otis Facility to evaluate Imidacloprid formulation samples using High Pressure Liquid Chromatography (HPLC) technology as a potential substitute for Immuno-Assay (IA) Kit analysis. ANPCL tasks included; (1) Establishment of experimental conditions to enable HPLC analysis for both purity and tank mix formulations and, (2) Analysis of comparative samples submitted from Otis used in ALB program pesticide applications in 2004.

- In less than 60 working days a robust HPLC alternative method was developed and 17 comparative samples were evaluated and reported.

Data was reported to CPHST scientists in Otis for comparison to existing technology used. Preliminary results are favorable for the conversion of analysis from IA kit to the HPLC technology. The net benefit for APHIS is the utilization of a more robust and cost effective tool for the evaluation and confirmation of formulations purchased, or mixed for use in the ALB program. Partners included: USDA, APHIS, PPQ, CPHST-Otis Scientists Vic Mastro and David R. Lewis.

Special CPHST Project: Determination of Bifenthrin in Multiple Soil Matrices using GC/ECD
Leader: Joyce James (In-progress, project started October 2004, expected duration through 2006)

High Pressure Liquid Chromatograph (HPLC) Preparative system--Used in Synthesis of pheromones

ANPCL was tasked with the evaluation of various control samples in multiple soil matrices to evaluate the net results of mixing efforts by nursery workers and monitor the degradation of Bifenthrin in soil mixes over a two year period. A method was developed using Gas Chromatograph (GC) with Electron Capture Detection (ECD).

- To date in 2004 a method was developed and the unit evaluated and reported results for 36 initial samples.

The preliminary data have proven useful in evaluating the program and unexpected benefits have been utilized in revising existing methods

to improve customer service. This project also includes a degradation study (re-sampling of designated soils over time) that will not be completed until early 2006. Partners include: USDA, APHIS, PPQ, CPHST, Soil Inhabiting Pests Section program leader Ann-Marie Callcott in support of the Imported Fire Ant (IFA) quarantine efforts to manage the spread of Fire Ants in nursery soils

Special CPHST Project:
ISO 17025 Implementation Leaders: R.D. Smith, Lead Chemists and Quality Management staff (On-going)

As part of our commitment to quality, ANPCL is in the process of implementing ISO-17025 based Quality Aspects for laboratory operations.

- In 2004 primary ISO documents and associated work instructions were drafted and introduced.
- The laboratory committed and continues to commit extensive time and effort in producing working ISO procedures that are based on trail and error adaptations of the initial documents.

The revision process continues with multiple evaluations and revisions of documents and procedures underway to obtain the Quality objective, while maintaining a robust technical service to our customers.

- In 2004, initial documents under ISO-17025 were completed in support of the existing Quality Manual.
- Multiple methods and primary customer support procedures were also established. With the advent of a CPHST Quality Director, ANPCL will continue under the direction of the program leader in implementation of ISO principles not in word, but through work effort and determination.

Any and all progress in this endeavor will directly benefit APHIS customer support and posture the laboratory for future work assignments. Partners include: USDA, APHIS, PPQ, CPHST - Director of Quality Management and staff.

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